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The patch-clamp and planar lipid bilayer techniques: powerful and versatile tools to investigate the CFTR Cl^- channel

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Abstract

Using the patch-clamp (PC) and planar lipid bilayer (PLB) techniques the molecular behaviour of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel can be visualised in real-time. The PC technique is a highly powerful and versatile method to investigate CFTR's mechanism of action, interaction with other proteins and physiological role. Using the PLB technique, the structure and function of CFTR can be investigated free from the influence of other proteins. Here we discuss how these techniques are employed to investigate the CFTR Cl^- channel with special emphasis on its permeation, conduction and gating properties.

Keywords: Chloride channel activity; Whole-cell recording; Single-channel recording; Anion permeation; Channel gating; Channel regulation

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR [1]) is a phosphorylation-regulated Cl^- channel that plays a central role in transepithelial fluid and electrolyte transport [2,3]. Like other members of the ATP-binding cassette (ABC) transporter superfamily, CFTR is assembled from two motifs each possessing a membrane-spanning domain (MSD) composed of six transmembrane segments and a nucleotide-binding domain (NBD) that interacts with ATP. Unique to CFTR is a regulatory (R) domain that contains multiple consensus phosphorylation sites and many charged amino acids. The MSDs assemble to form the Cl^- selective pore while the NBDs and R domain tightly control CFTR channel gating (for review, see Refs. [2,3]).

Abbreviations: ABC transporter, ATP-binding cassette transporter; CA membrane patch, cell-attached membrane patch; $I–V$ relationship, current–voltage relationship; CFTR, cystic fibrosis transmembrane conductance regulator; 8-CPT-cAMP, 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; DPC, diphenylamine-2-carboxylate; EBIO, 1-ethylbenzimidazolone; EIO membrane patch, excised inside-out membrane patch; GHK equation, Goldman–Hodgkin–Katz equation; G_m, membrane conductance; IMBX, 3-isobutyl-1-methylxanthine; MSD, membrane-spanning domain; V_m, membrane voltage; P, permeability; PC technique, patch-clamp technique; PKA, protein kinase A; PLB technique, planar lipid bilayer technique; P_o, open probability; NBD, nucleotide-binding domain; PE, phosphatidyleth-anolamine; PS, phosphatidylserine; R domain, regulatory domain; SC, single-channel; WC, whole-cell; WT, wild-type; VIP, vasoactive intestinal polypeptide.

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Much of our knowledge and understanding of the CFTR Cl^- channel originates from studies using the patch-clamp (PC) and planar lipid bilayer (PLB) techniques [4,5]. What makes these techniques so powerful is their ability to resolve current flow through individual CFTR Cl^- channels in real-time. A further strength of the PC technique is its versatility: current flow through either single or multiple CFTR Cl^- channels can be investigated in intact cells and in cell-free membrane patches. Moreover, using the PLB technique structure–function relationships can be examined unhindered by interacting proteins. The website of the European Working Group on CFTR Expression provides a variety of protocols to investigate the CFTR Cl^- channel using the PC and PLB techniques [6]. Here we provide an overview of these different protocols to study the macroscopic and single-channel (SC) properties of CFTR.

2. Whole-cell recording

The whole-cell (WC) configuration of the PC technique assesses the activity of all active ion channels in the plasma membrane of a cell under precisely defined ionic conditions [4]. Moreover, with appropriate care, intracellular signalling cascades are preserved allowing the regulation of ion channels by physiologically important signals to be assessed [7]. Given that CFTR interacts closely with a rich variety of proteins [8], WC recording is an ideal strategy to investigate the overall function of CFTR in epithelial cells.

Using the WC configuration, CFTR can be investigated in two ways. In the current-clamp mode, the influence of CFTR on membrane voltage is evaluated. In contrast, current flow through CFTR Cl^- channels is measured in the voltage-clamp mode.

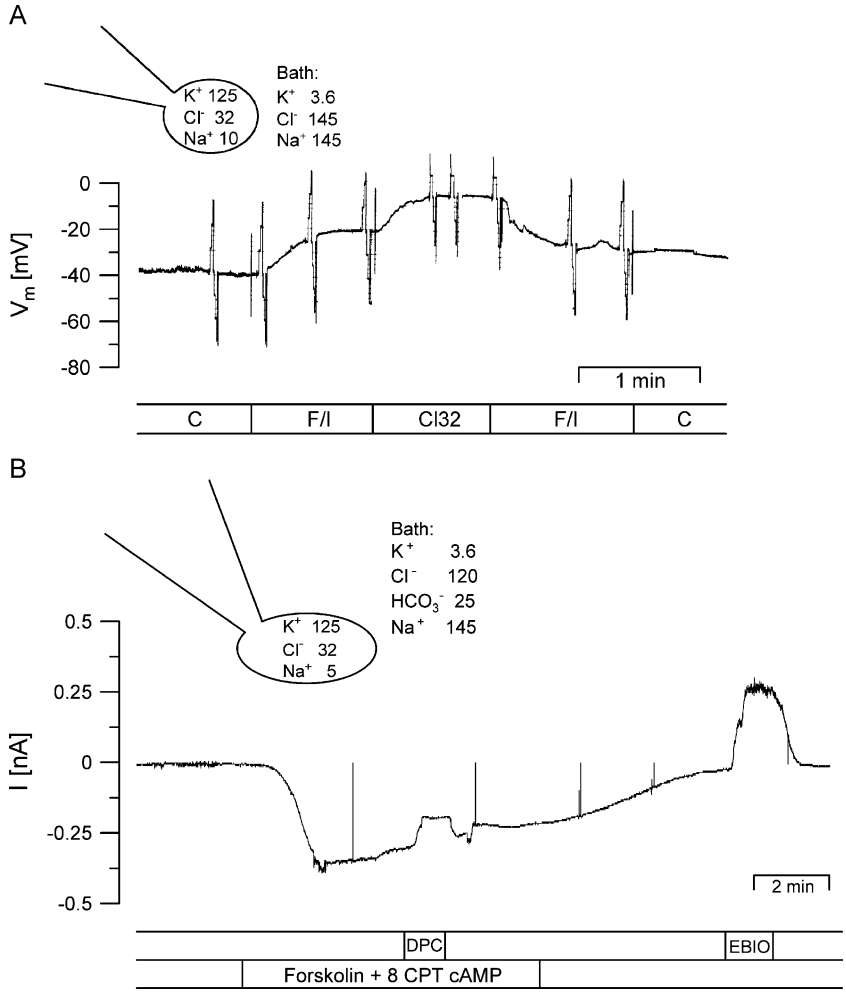

Fig. 1. WC recordings of CFTR activity in current (A)- and voltage (B)-clamp modes. (A) Time-course of membrane voltage recorded from a CHO cell expressing wt human CFTR current-clamped at 0 pA. Abbreviations: C, control, F/I, forskolin (0.1 μM) + 3-isobutyl-1-methylxanthine (IMBX; 100 μM); Cl32, 32 mM Cl^- extracellular solution. The vertical lines indicate times during which the PC amplifier was briefly switched to the voltage-clamp mode to acquire *I–V* relationships. The icon in the upper left-hand corner describes the composition (mM) of the bath and pipette solutions, respectively. Modified, with permission, from Ref. [29]. (B) Time-course of WC current recorded from a Calu-3 cell voltage-clamped at −40 mV. During the periods indicated by the bars forskolin (10 μM) and 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate (8 CPT-cAMP; 100 μM), diphenylamine-2-carboxylate (DPC; 1 mM) and 1-ethylbenzimidazolone (EBIO; 1 mM) were present in the extracellular solution. The vertical lines indicate times during which the PC amplifier was briefly switched to current-clamp mode to measure V_m. Other details as in A.

2.1. Measurement of membrane voltage using the current-clamp mode

Membrane voltage (V_m) is determined by (i) the identity and number of active ion channels in the cell membrane and (ii) by the ionic composition on either side of the membrane. If this information is known, V_m can be calculated using the Goldman–Hodgkin–Katz (GHK) equation [9]. One way of writing the GHK equation is:

$$V_m = (f_K \times E_K) + (f_{Na} \times E_{Na}) + (f_{Cl} \times E_{Cl}) + \ldots, \quad (1)$$

where f_X is the fractional conductance of the membrane for the respective ion and E_X is the corresponding Nernst (equilibrium) voltage (for typical values of E_X and f_X, see Ref. [6]). To discern the contribution of CFTR to the membrane voltage of a cell, V_m is measured at a holding current of 0 pA. If V_m follows E_{Cl} when E_{Cl} is manipulated experimentally, this would suggest that a Cl^- conductance such as CFTR mediates the changes in membrane voltage (see Fig. 1A and Ref. [6]).

2.2. Measurement of WC currents using the voltage-clamp mode

The voltage-clamp mode is the WC method routinely used to assess CFTR function. When a voltage is applied across a cell membrane, the current is dependent upon the resistance of the patch–pipette—cell network (see Ref. [6]). Stimulation of membrane resident CFTR channels with cAMP agonists will increase their open probability (P_o) and hence, increase membrane conductance (G_m). The resulting CFTR Cl^- current is monitored at a constant holding (-clamp) voltage. According to the established sign convention, a flow of positive ions (cations) out of the cell (anions moving in the opposite direction) is defined as a positive (outward) current, whereas the converse is termed a negative (inward) current. For example, Fig. 1B shows a WC recording with both inward and outward currents. Activation of CFTR with cAMP agonists generated an inward (Cl^-) current that reversed on washout of the agonists. Diphenylamine-2-carboxylate (DPC, 1 mM) partially inhibited CFTR leading to a reduction of the inward current. In contrast, stimulation of K^+ channels with 1-ethylbenzimidazolone (EBIO, 1 mM) generated an outward (K^+) current.

If the holding voltage is close to the actual membrane voltage of the cell, little, if any, change in WC current will occur upon activation of CFTR with cAMP agonists. As a result, most experimental protocols include predefined voltage-ramps or -steps that change the holding voltage in a ramp- or step-like manner (for example protocols, see Ref. [6]). The resulting current–voltage (I–V) relationship can be utilized to calculate the total WC CFTR Cl^- conductance and to examine the selectivity of CFTR Cl^- currents. Voltage protocols also provide important information about the regulation of CFTR Cl^- currents including the time- and voltage-dependence of CFTR. For further information see Ref. [6].

3. Single-channel recording

Single-channel (SC) studies provide a molecular explanation for quantitative changes in macroscopic CFTR Cl^- currents recorded using the WC configuration of the PC technique. The SC activity of CFTR can be studied using three different configurations of the PC technique: (i) cell-attached (CA), (ii) excised inside-out (EIO) and (iii) excised outside-out membrane patches. Studies using the first two configurations have provided a wealth of information about the CFTR Cl^- channel [2,3]. In contrast, excised outside-out membrane patches are very rarely used to investigate CFTR; they will therefore not be considered in this article.

4. Cell-attached membrane patches

The CA configuration of the PC technique is used to study the properties and regulation of either single or more often small numbers of CFTR Cl^- channels. The major advantage of this recording configuration is that the membrane patch remains part of an intact cell throughout the course of the experiment. As a result, CFTR Cl^- channels are studied in their native environment with the inside face of the membrane patch remaining in contact with the cytoplasm of the cell. Thus, CA patches represent an ideal way to investigate the SC activity of CFTR under as near normal physiological conditions as possible.

Following seal formation, spontaneous openings of the CFTR Cl^- channel are frequently observed (Fig. 2, top trace). This low-level, basal activity of CFTR likely represents endogenously phosphorylated CFTR Cl^- channels [2].

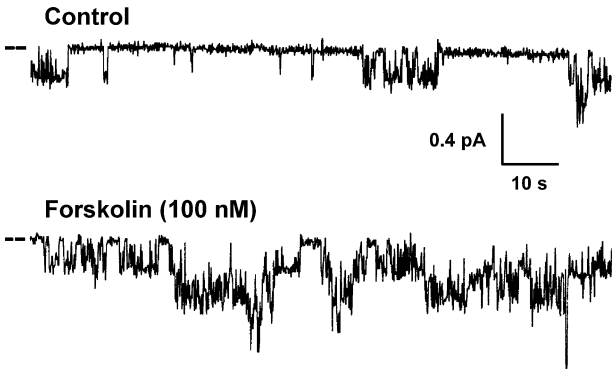

Fig. 2. Activation of CFTR Cl^- channels by forskolin in a CA membrane patch. Representative recordings of CFTR Cl^- channels in a CA membrane patch on a cultured human pancreatic duct cell. Each trace is 50 s long recorded at a pipette voltage (V_{cmd}) of 62 mV. The top trace was recorded under control conditions and the bottom 2 min after the addition of forskolin (100 nM) to the extracellular solution. The dashed line on the left indicates the current level when all the channels were closed. Downward deflections of the trace correspond to channel openings. Both the bath and pipette solutions were NaCl-rich. Modified, with permission from Ref. [30].

CFTR activity is markedly enhanced by exposing cells to either physiological hormones or neurotransmitters that elevate the intracellular concentration of cAMP (e.g. vasoactive intestinal polypeptide, VIP; adenosine; noradrenaline). By far the most effective and reproducible method of activating CFTR in CA membrane patches is to expose cells to a cocktail of cAMP agonist and perform experiments at 30–37 °C [6]. Of note, Huang et al. [10] demonstrated that addition of adenosine to the pipette solution, by itself, activated CFTR Cl^- channels in CA membrane patches from the human airway epithelial cell line Calu-3. These data provide compelling evidence that all of the machinery required to regulate CFTR are co-localised in a molecular signalling complex at the apical membrane of airway epithelial cells.

There are two drawbacks of the CA configuration: first, the true membrane voltage of the patch is unknown and second, the composition of the intracellular solution (the cytoplasm) is uncertain and cannot be manipulated experimentally.

The membrane voltage across the patch (V_{patch}) is unknown because both the resting membrane voltage of the cell (V_{cell}) and the voltage applied to the inside of the pipette (V_{cmd}) both contribute:

$$V_{patch} = V_{cell} - V_{cmd}, \quad (2)$$

In other words, if no voltage is applied to the interior of the patch-pipette ($V_{cmd} = 0$), then the CA patch will be at the normal resting V_m of the cell. Physiologically, this is the most important condition in which to study the CFTR Cl^- channel. However, using only one voltage, and not knowing it precisely, limits severely the amount of data that can be acquired. Compounding this problem, it is difficult to resolve individual openings of CFTR Cl^- channels at the resting V_m. To optimise the recording conditions, V_{patch} should be changed by applying depolarising (i.e. negative V_{cmd}) and/or hyperpolarizing (i.e. positive V_{cmd}) voltages to the interior of the patch-pipette. To calculate V_{patch}, an estimate of V_{cell} is required. One approach is to establish the WC configuration at the end of the experiment and measure membrane voltage in the current clamp mode at 0 pA. For accurate V_{cmd} measurements, the pipette solution should mimic that of the cytoplasm of the cell. (Other protocols to determine V_{cell} are discussed in Ref. [6]).

The second major limitation of the CA configuration is that the composition of the intracellular solution is not precisely known, nor can it easily be altered. Furthermore, the extracellular (pipette) solution, while controllable, is essentially fixed for the duration of the experiment. This hampers, but does not prevent, studies of CFTR selectivity and pharmacology. Indeed, using CA membrane patches, Gray et al. [11] and Tabcharani et al. [12] demonstrated that disulphonic stilbenes (e.g. 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid, DIDS) are without effect on the CFTR Cl^- channel when present on the extracellular side of the membrane.

Thus, the CA configuration is the method of choice to investigate the behaviour of individual CFTR Cl^- channels under normal physiological conditions. In contrast, EIO membrane patches are preferred to investigate the relationship between the structure and function of the CFTR Cl^- channel.

5. Excised inside-out membrane patches

Several factors make EIO membrane patches uniquely suitable for studies of CFTR structure and function. First, they permit precise control of the composition of the solutions bathing the intra- and extracellular sides of the membrane. Second, V_m is known and can be controlled accurately without the need for series resistance compensation. Third, regulation of CFTR by cAMP-dependent phosphorylation of the R domain and ATP binding and hydrolysis at the NBDs can be tightly controlled and readily manipulated during experiments by changing the composition of the bath (intracellular) solution. Fourth, when compared with CA membrane patches contaminating electrical and mechanical noise is often considerably lower in EIO membrane patches. Finally, SC properties can be investigated directly in EIO membrane patches.

Depending on (i) the level of CFTR expression in the cell selected for study and (ii) the geometry of the patch-pipette employed, a membrane patch might contain anything from a single active CFTR Cl^- channel to hundreds, even thousands of CFTR Cl^- channels. Membrane patches with only a single active CFTR Cl^- channel are highly prized because they provide the rare opportunity to examine gating kinetics [6]. In contrast, macroscopic current recording is employed either when SC current amplitude is below the limit of resolution or when the average response of a large number of channels is required [6]. In either case, CFTR Cl^- currents are identified by their absolute dependence on protein kinase A (PKA) and ATP in the intracellular solution, characteristic slow gating that is time- and voltage-independent and by current potentiation by the inorganic phosphate analogue pyrophosphate (PP_i), which increases both the frequency and duration of channel openings [6].

EIO membrane patches have been used extensively to examine the biophysical properties, regulation and pharmacology of the CFTR Cl^- channel. Strategies to investigate the mechanism of action of CFTR modulators are discussed in Ref. [6]. Below, we discuss studies of CFTR permeation, conduction and gating kinetics using membrane patches containing either multiple or single CFTR Cl^- channels. Note that high quality recordings are essential to quantify accurately CFTR behaviour (for discussion, see Ref. [6]).

5.1. Measurement of the permeation and conduction properties of CFTR

Anion selectivity is most easily studied under biionic conditions. That is, with Cl^- present as the only permeant anion on one side of the membrane, and another anion present at the same concentration on the opposite side. This approach is equally applicable to macroscopic [13] and SC currents [14]. In both cases, the critical parameter is the zero current voltage (reversal potential) obtained from $I–V$ relationships. Under strictly biionic conditions (and assuming zero cation permeability), with monovalent anions A^- in the intracellular solution and B^- in the extracellular solution, the relative permeability (P) of these two anions is given by a modified form of the GHK voltage equation [9]:

$$P_A/P_B = \exp(E_{rev}F/RT), \quad (3)$$

where E_{rev} is the estimated current reversal potential, F is the Faraday constant (9.648×10^4 C mol^{-1}), R is the gas constant (8.314 J mol^{-1} K^{-1}) and T is the temperature (in Kelvin). Because relative permeability is calculated, the permeability of "foreign" anions is estimated relative to that of Cl^-. With Cl^- in the intracellular solution and anion X^- in the extracellular solution:

$$P_X/P_{Cl} = \exp(-E_{rev}F/RT), \quad (4)$$

and with Cl^- in the extracellular solution and anion X^- in the intracellular:

$$P_X/P_{Cl} = \exp(E_{rev}F/RT), \quad (5)$$

This assumes a reversal potential of zero with symmetrical Cl^- rich solutions. For further information about anion permeation measurements, see Ref. [6].

In EIO membrane patches, the CFTR Cl^- channel has a linear $I–V$ relationship at negative voltages, but a weakly inwardly rectifying $I–V$ relationship at positive voltages [15]. At negative voltages, the SC conductance of wild-type (wt) human CFTR is about 8 pS in the presence of symmetrical Cl^- -rich solutions [3]. However, certain ionic conditions [13,16] and some mutations within the channel pore [16] reduce the SC conductance of CFTR below the limit of resolution using the PC technique. In these cases, SC conductance can be estimated by noise analysis of macroscopic CFTR Cl^- currents (Fig. 3 and Ref. [17]). Thus, the relative conductance of different anions can be estimated using either SC or macroscopic CFTR Cl^- currents by bathing excised patches symmetrically in the test anion provided that the test anion generates large enough currents to resolve SC openings. Using either method, the same conductance sequence is obtained (Fig. 3).

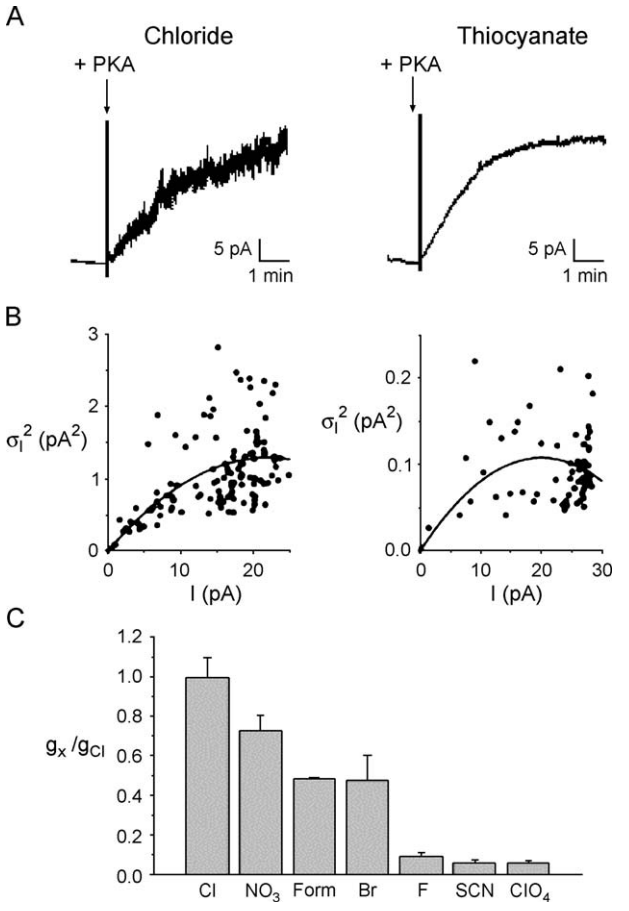

Fig. 3. Anion conductance of CFTR determined by current variance analysis. (A) Slow activation of macroscopic CFTR Cl^- currents in EIO membrane patches from BHK cells expressing wt human CFTR by the addition of PKA (20 nM) in the presence of 1 mM ATP at a membrane voltage of +50 mV. Left, symmetrical 150 mM Cl^- solutions and right, symmetrical 150 mM SCN^- solutions. (B) Relationship between CFTR current amplitude (I) and current variance (σ_I^2) for the data shown in A. Other details as in Ref. [16]. (C) Anion conductance measured under symmetrical conditions using macroscopic current variance analysis. Form, formate. Data are means ± S.E.M. ($n = 3–9$). Reproduced, with permission from Ref. [16].

Because many different anions permeate the CFTR pore, information about the physical properties of anions provides insight into the physical principles that underlie anion–channel interactions [6]. In particular, the free energy of hydration of anions (the energy required to remove the waters of hydration from anions in solution) correlates with the permeation properties of CFTR by following a lyotropic sequence [18]. Lyotropic anions (e.g. SCN^-), which are relatively easily dehydrated, show both higher relative permeability and tighter binding within the CFTR pore [18]. Similarly, information about the geometry of the CFTR pore has been derived from the dimension of dehydrated anions. These measurements suggest that the narrowest part of the CFTR pore is ~ 0.53–0.60 nm in diameter [14], widening under certain circumstances to a diameter of ~ 1.3 nm [13].

To define the molecular architecture of the CFTR pore, CFTR variants bearing site-directed mutations are employed [19]. When mutation of a particular amino acid residue alters anion permeation, the nature of the anion–amino acid interaction can be examined by substituting multiple amino acids that differ in side chain size, charge and polarity (e.g. Ref. [16]). Importantly, as with all mutagenesis studies, there are important caveats: mutations within the CFTR pore might cause indirect and potentially global changes in pore architecture (e.g., R347D, [20]). Alternatively, mutations might disrupt channel gating (e.g., R352Q, [21]).

5.2. Kinetic analysis of single CFTR Cl^- channels

Visual inspection of SC records (Figs. 2 and 4) suggests that there are at least two populations of channel closures. Openings of the CFTR Cl^- channel occur in bursts separated by long closures in the range of hundreds of ms to s.

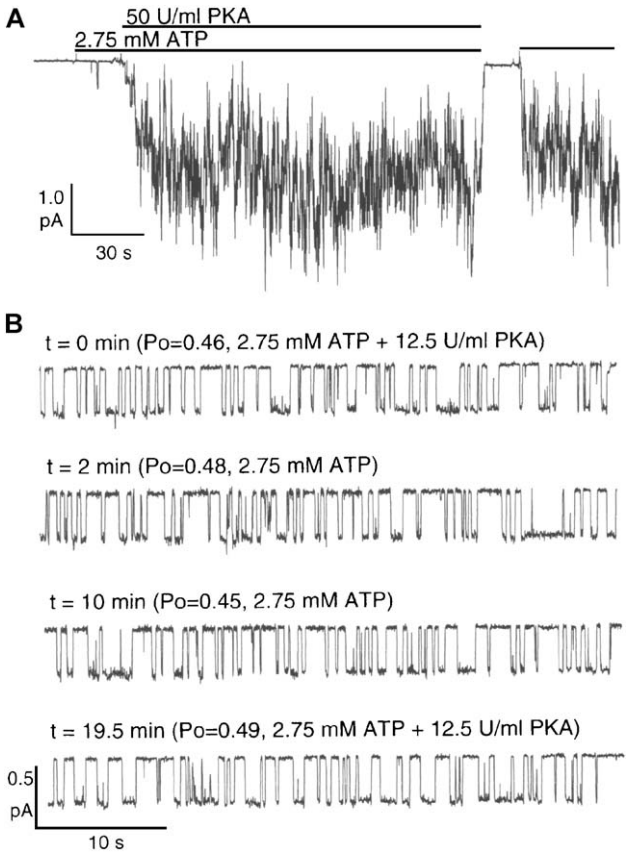

Fig. 4. Intracellular ATP is required to open PKA phosphorylated CFTR Cl^- channels in EIO membrane patches. (A) Time-course of CFTR current in an EIO membrane patch. During the periods indicated by the bars ATP (2.75 mM) and PKA (50 U/ml) were present in the intracellular solution. (B) A semi-continuous recording of a single CFTR Cl^- channel under the indicated conditions. Downward deflections of the traces correspond to channel openings. The open probability (P_o) for each 45 s trace is indicated. Reproduced from The Journal of General Physiology 1999, 113, 541–554 by copyright permission of The Rockefeller University Press.

Within each individual burst, there are brief flickery closures of several to tens of ms in duration.

To investigate the gating kinetics of the brief flickery closures interrupting channel openings, Zhou et al. [22] used K1250A, a CFTR construct with an elevated P_o ($P_o \sim 0.9$), and a simple model of channel block:

$$\text{Open} \underset{\beta}{\overset{\alpha}{\leftrightarrow}} \text{Blocked}, \tag{6}$$

where α is the apparent on-rate of the blocker and β the off-rate. The authors showed that the blocked-time, but not the open-time, is voltage-dependent and sensitive to the concentration of permeant anions in the external solution [22]. Based on these data, Zhou et al. [22] proposed that within the CFTR pore, a permeant anion entering the pore from the extracellular side electrostatically repels the blocker entering from the intracellular side.

To investigate ATP-dependent channel gating, SC records are heavily filtered prior to analysis. For example, Zeltwanger et al. [23] demonstrated that filtering data at 25 Hz during playback and digitisation had little or no effect on the slow ATP-dependent gating events, but almost completely eliminated the brief flickery closures. The authors further showed that open bursts are defined as openings separated by closures lasting longer than 80 ms [23]. Analysing data in this way, in the presence of saturating ATP concentrations mean open- and closed-times are in the range of hundreds of ms in excellent agreement with data obtained from analyses of macroscopic CFTR Cl^- currents [24]. Interestingly, Zeltwanger et al. [23] showed that the closed-time histogram of single CFTR Cl^- channels contains a negative exponential component, suggesting that CFTR gating is not in equilibrium. The presence of an irreversible step in CFTR gating suggests that channel gating might be strictly coupled to ATP hydrolysis. If this is indeed the case, the data of Zeltwanger et al. [23] predict that CFTR hydrolyses ATP at a rate of $\sim 1\ s^{-1}$ in close agreement with the results of Li et al. [25].

6. Planar lipid bilayers

Like the PC technique, the PLB technique visualises conformational changes in individual CFTR Cl^- channels in real-time. However, measurement of channel activity using PLBs necessitates that CFTR protein must first be harvested and fused with an artificial lipid membrane [26]. Frequently, membrane vesicles from either mammalian cells or *Xenopus* oocytes expressing recombinant CFTR are used as a source of CFTR protein [6]. However, the best material to use is purified recombinant CFTR protein reconstituted into liposomes [26,27]. Using purified recombinant CFTR reconstituted into PLBs, functions can be unambiguously attributed to CFTR.

The fusion and detection of CFTR-containing liposomes with the PLB is achieved by the addition of nystatin, a channel-forming peptide to liposomes of the following composition: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine and ergosterol in the ratio 5:2:1:1. Nystatin can only fuse with the bilayer and form an active channel in the presence of ergosterol or cholesterol [28]. Following fusion, which is marked by a transient conductance spike, ergosterol diffuses from the bilayer, which is composed of only PS and PE followed by dissipation of the nystatin channel. As a result CFTR Cl^- channels remain fused to the PLB where they can be studied, in isolation, using SC recording techniques.

7. Conclusion

The PC and PLB techniques have played a key role in the acquisition of knowledge and understanding of the CFTR Cl^- channel. They have been instrumental in (i) understanding the complex relationship between the structure and function of CFTR, (ii) the role of CFTR in transepithelial ion transport, (iii) elucidating the different mechanisms by which CF mutations cause a loss of Cl^- channel function and (iv) the discovery of new drugs to treat CF. We expect fully these techniques to remain crucial for CFTR research for many years to come.

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